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Heparan Sulfate-Mediated Binding of Infectious Dengue Virus Type 2 and Yellow Fever Virus

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Dengue virus type 2 and Yellow fever virus are arthropod-borne flaviviruses causing hemorrhagic fever in humans. Identification of virus receptors is important in understanding flavivirus pathogenesis. The aim of this work was to study the role of cellular heparan sulfate in the adsorption of infectious Yellow fever and Dengue type 2 viruses. Virus attachment was assessed by adsorbing virus to cells, washing unbound virus away, releasing cell-bound virus by freezing/thawing, and then titrating the released infectious virus. Treatment of cells by heparin-lyase, desulfation of cellular heparan sulfate, or treatment of the virus with heparin inhibited cell binding of both viruses. Heparin also inhibited Yellow fever virus infection by 97%. Using infectious virus, the present work shows the importance of heparan sulfate in binding and infection of these two flaviviruses.

Key Words: flaviviruses; heparan sulfate; receptor; binding; glycosaminoglycan.

INTRODUCTION

Dengue (DEN) viruses and Yellow fever (YF) virus are arthropod-borne flaviviruses that cause hemorrhagic fever (associated with hepatitis) in humans (Henchal *et al.,* 1990; Monath and Heinz, 1996). These viruses belong to the family of the *Flaviviridae*, which also includes hepatitis C virus (HCV, genus *Hepacivirus*). The genomes of YF virus and DEN viruses are single-stranded, positive-sense RNA molecules coding for the structural proteins (core, membrane (M), and envelope (E)) in the 5′ quarter of the genome and for eight nonstructural proteins in the remainder of the coding sequence (Rice *et al.,* 1985). Mosquito-borne YF and DEN viruses are responsible for an important public health problem caused, among other things, by the failure to maintain programs for controlling the mosquito vector, *Aedes aegyptii.* YF diseases are increasing and some 208 cases occurred in 1999, principally in South America, with a 49% of mortality rate (WHO Weekly Epidemiologic Record, 2000). Although the virus can be controlled by the use of an efficient live vaccine, serious adverse side effects were recently reported by Marianneau *et al.* (2001). In contrast, DEN viruses are endemic in (sub)tropical areas and affect one million individuals each year, with a high mortality rate in children (Monath and Heinz, 1996). Furthermore, one outbreak of Dengue occurred in the United States in 1995 (CDC Weekly Report, 1996). There is no specific treatment for flavivirus infections.

The binding of a virus to its specific receptor(s) is a major factor of cellular tropism and a critical determinant of pathogenesis. Although some work has been carried out on DEN virus binding (Ramos-Castaneda *et al.,* 1997; Munoz *et al.,* 1998; Bielefeldt-Ohmann, 1998; Hung *et al.,* 1999), the initial event of flavivirus infection has not been described in detail and little is known about the molecular basis of the binding of flaviviruses to their target cells. Heparan sulfate (HS) (the most ubiquitous member of molecules of the glycosaminoglycan (GAG) family) is used by many viruses to bind to target cells, such as herpes simplex virus (Shukla *et al.,* 1999), human immunodeficiency virus (Patel *et al.,* 1993), foot and mouth disease virus (Fry *et al.,* 1999), vaccinia virus (Lin *et al.,* 2000), adenoassociated virus 2 (Summerford and Samulski, 1998), Sindbis virus (Byrnes and Griffin, 1998; Klimstra *et al.,* 1999), papillomavirus (Joyce *et al.,* 1999), respiratory syncytial virus (Feldman *et al.,* 2000; Hallak *et al.,* 2000), adenovirus types 2 and 5 (Dechecchi *et al.,* 2001), and Echovirus (Goodfellow *et al.,* 2001). HS could act directly as a receptor or help to concentrate these viruses on the cell surface to facilitate the interaction with specific high-affinity receptors. Recently, Chen *et al.* (1996, 1997) showed that a soluble, recombinant and hybrid surface glycoprotein of DEN-2 virus bound to highly sulfated GAGs on the surface of Vero cells and

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TABLE 1

Development of a Virus-Binding Assay

Virus	Inoculum dilution	$TCID_{50}$/well ($\times 10^{-3}$) binding set	$TCID_{50}$/well ($\times 10^{-6}$) replicating set
DEN-2 virus	Undiluted	7.1 ± 0.6	4.6 ± 0.2
	Diluted 3 times	2.0 ± 0.2	1.6 ± 0.3
	Diluted 10 times	0.36 ± 0.03	0.34 ± 0.07
YF virus	Undiluted	8.9 ± 1.2	15.4 ± 2.3
	Diluted 3 times	2.0 ± 0.2	6.8 ± 1.2
	Diluted 10 times	0.7 ± 0.1	1.1 ± 0.2

Note. The binding assay (see Materials and Methods) was performed with different virus dilutions (1, 1/3, 1/10). For the binding set, cells were collected after 15 min. For the replicating set, cells were cultured for 48 h (DEN-2 virus) or for 36 h (YF virus) before collection and virus titration. The undiluted inoculum contained $10^{4.6}$ $TCID_{50}$/well for DEN-2 virus and $10^{4.9}$ $TCID_{50}$/well for YF virus.

that infection of these cells could be prevented by heparin and by high-sulfate HS.

Instead of the binding of recombinant proteins to cells, the present work studies the role of HS in binding of infectious DEN-2 viruses to the surface of CHO and Vero cells. It also shows for the first time that YF virus also binds to HS on the surface of Vero cells and that this is a prerequisite for infection. Heparin can inhibit YF virus infection of Vero cells by up to 97%.

RESULTS

Development of a virus binding assay

For the validation of the binding assay, three virus dilutions (undiluted and 3 times and 10 times diluted) were used to inoculate two sets of Vero cells. The undiluted inoculum contained $10^{4.6}$ 50% tissue culture infectious doses per well ($TCID_{50}$/well) for DEN-2 virus and $10^{4.9}$ $TCID_{50}$/well for YF virus (Table 1). After 15 min the cells were washed to remove unbound virus. Then one set (called binding set) was immediately collected and the infectious virus that was bound to the cells was released by freezing/thawing and titrated. Less than 1% of infectivity remained associated with the pellet of the cell debris. The other set (called replicating set) was incubated with medium for 48 h (DEN-2 virus) or 36 h (YF virus) before freezing/thawing and titration of released virus. The results shown in Table 1 indicate that the titers of virus released from the cells of the binding set and the replicating set were proportional to the dilution factors of the inoculum, although the titers for the replicating set were about 1000 times higher than those for the binding set. A Spearman's test demonstrated the correlation between the results obtained with the two sets of cells (Rho $>$ 0.9, $P < 0.01$). We will subsequently use the assay described as the binding set to study the effects of GAG modifications on the binding of DEN and YF viruses.

DEN-2 virus and YF virus bind to specific, sulfated GAG species on the surface of cells

GAG-lyases remove GAGs from cells. Attachment of a DEN-2 recombinant E glycoprotein to Vero cells was reduced when GAGs were enzymatically removed (Chen *et al.,* 1997). To demonstrate that GAGs play a role in the binding of infectious DEN-2 and YF viruses to the target–cell surface, Vero cells were treated for 1 h at 37°C with heparin-lyase I (specific for highly sulfated domains of HS and heparin) and chondroitin ABC-lyase (specific for chondroitin and dermatan sulfate). Heparin-lyase I significantly inhibited binding of both flaviviruses (Fig. 1A) but chondroitin ABC-lyase had no effect. This enzyme specificity implies that the viruses bind specifically to HS.

For most of the heparin-binding viruses, sulfation of the cell surface is critical for virus binding and infection. Addition of sodium chlorate to sulfate-free medium blocks cellular ATP-sulfurylase and sulfate adenyl transferase activities and reduces incorporation of sulfate into GAGs (Baeuerle and Hunter, 1986). Cells grown in this type of medium had a severely reduced capacity to bind DEN-2 and YF viruses (less than 20% of the untreated control) (Fig. 1B). As a control, cells treated as described above were supplemented with sodium sulfate, which reversed the effect of the sodium chlorate and led to partial restoration of virus binding.

Binding of DEN-2 virus to Chinese hamster ovary (CHO) cells defective in GAG expression

If cell surface GAGs are involved in flavivirus adsorption, cell lines deficient in enzymes needed to produce the various GAGs might differ in their susceptibility to bind flaviviruses. Mutant cell lines derived from CHO K1 are psgB-618, which is defective for galactosyltransferase-I, resulting in the inhibition of both chondroitin and HS synthesis, and psgD-677, which is defective for HS polymerization and expression, but has an increased expression of other GAGs (Esko *et al.,* 1985, 1987; Lidholt *et al.,* 1992). DEN-2 virus binding to the two mutant cell lines was reduced by more than 75% compared to binding to wild-type CHO K1, with no significant difference between the two mutants (Fig. 2). YF virus adsorption to CHO cells was too low to be studied.

Heparin inhibits Vero cell binding by DEN-2 and YF viruses

If the presence of HS on the cell surface influences flavivirus binding, then soluble HS might also interfere with virus adsorption. Heparin has the same carbohydrate composition as HS except that it contains more iduronic acid (the epimerized form of glucuronic acid), is more highly sulfated, and is less acetylated (Lindahl *et*

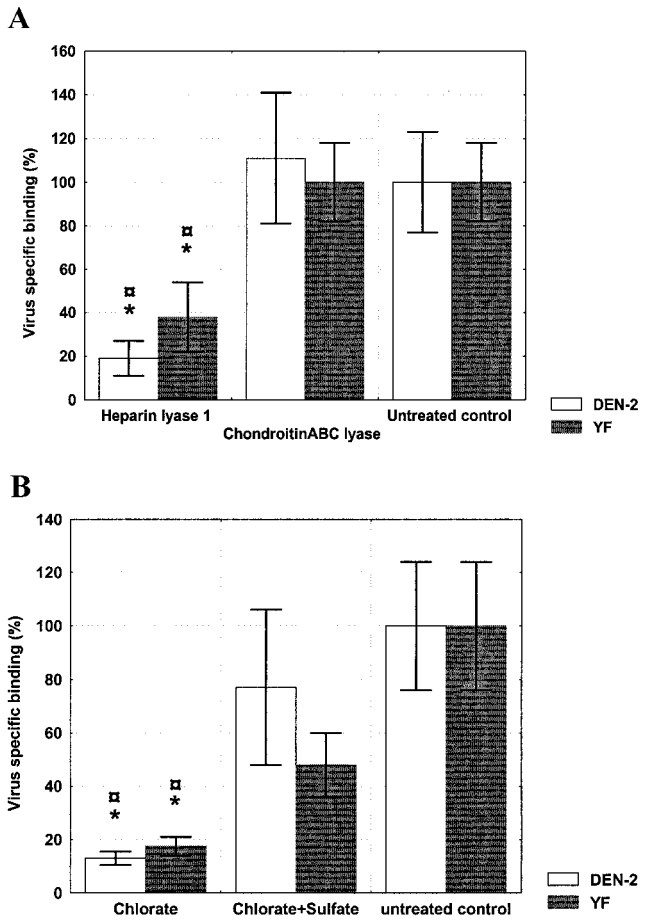

FIG. 1. Effect of GAG-lyase treatment and GAG desulfation on DEN-2 and YF virus adsorption to cells. (A) Binding of DEN-2 (open bars) and YF (shaded bars) virus to Vero cells after treatment of the cells with heparin-lyase I or chondroitin ABC-lyase. The DEN-2 virus titer of the untreated control was $10^{3.2}$ $TCID_{50}$/well and the YF virus titer of the untreated control was $10^{3.2}$ $TCID_{50}$/well. (B) Binding of virus to Vero cells in the presence of the sulfation inhibitor sodium chlorate, with or without sodium sulfate which restored sulfation. Sulfated and nonsulfated cells were inoculated with DEN-2 (open bars) or YF (shaded bars) viral suspensions for the binding assay. The DEN-2 untreated control was $10^{3.1}$ $TCID_{50}$/well and the YF untreated control was $10^{3.3}$ $TCID_{50}$/well. Titers were calculated in $TCID_{50}$/well and results are presented in percentage of untreated control. The mean values and the standard deviations correspond to a quadruplicate assay. This experiment was repeated two times (GAG-lyases) or three times (desulfation) with similar results. *Student's *t* test for the difference between cells treated with heparin-lyase and nontreated cells or cells treated with sodium chlorate and untreated cells, $P < 0.05$. ¤Student's *t* test for the difference between cells treated with heparin-lyase I and chondroitin-lyase or cells treated by sodium chlorate or with sodium chlorate and sulfate, $P < 0.05$.

al., 1989; Lindahl, 1990). Heparin is often used experimentally as an HS analog. After incubation with increasing concentrations of heparin for 1 h at 37°C, attachment of DEN-2 (Fig. 3A) and YF (Fig. 3B) viruses was reduced in a dose-dependent manner. Maximum inhibition was between 60 and 70% and the 50% inhibitory dose (ID_{50}) was around 0.2 μg/mL for both viruses. The effect of exogenous chondroitin sulfate B on cell attachment was also determined to assess the specificity of the heparin–virus interaction. In agreement with the result shown in Fig. 1A, 200 μg/mL chondroitin sulfate B (corresponding to the highest dose of heparin) did not inhibit binding of either of the viruses (data not shown).

Heparin inhibits infection of Vero cells by YF virus

Chen *et al.* (1997) have shown that DEN-2 virus infection was inhibited by heparin and high-sulfate HS (maximum inhibitions of 99 and 87%, respectively). Therefore, in a final experiment, we tested the inhibition of infection of YF virus by heparin. Figure 4 shows that soluble heparin indeed reduced the production of infectious YF virus from $10^{6.3}$ to $10^{4.7}$ $TCID_{50}$/well (97% inhibition) with an ID_{50} of about 0.2 μg/mL.

DISCUSSION

HS is important for cell adsorption of recombinant DEN-2 virus E glycoprotein and for virus infection (Chen *et al.,* 1997). In the present work we have directly quantified the binding of infectious DEN-2 and YF virus to Vero cells. We have shown that binding of both flaviviruses to cells was strongly and specifically reduced when HS was desulfated or enzymatically removed from the cell surface. Moreover, heparin but not chondroitin sulfate reduced virus binding. Heparin was also able to inhibit the infection process of YF virus as was shown before for DEN-2 virus (Chen *et al.,* 1997; Hung *et al.,* 1999). These two groups have determined the ID_{50} of heparin for DEN virus infection and for binding of recombinant DEN glycoprotein to cells. These ID_{50} values were similar to the

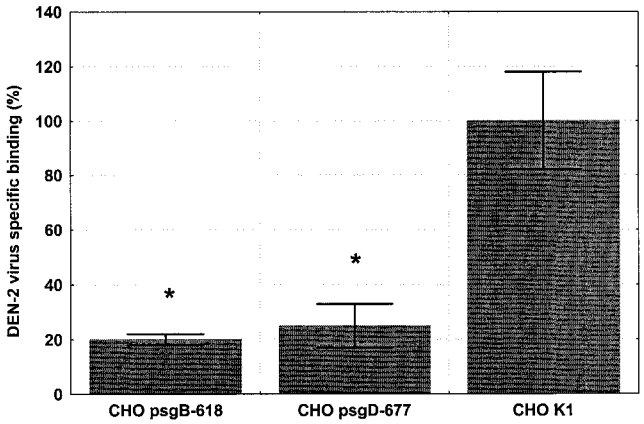

FIG. 2. DEN-2 virus binding to wild-type and mutant CHO cells. Binding of DEN-2 virus to wild-type CHO cells and two defective mutants lacking both heparan and chondroitin sulfate (CHO pgsB-618 defective in galactosyltransferase I) or lacking heparan sulfate only (CHO psgD-677 defective in heparan sulfate polymerization). Titers were calculated in $TCID_{50}$/mL and results are presented as percentages of the untreated control. The DEN-2 untreated control was $10^{2.4}$ $TCID_{50}$/well. The mean values and the standard deviations represent four independent assays. *Student's *t* test for the difference between wild-type CHO cells and defective mutant CHO cells, $P < 0.05$.

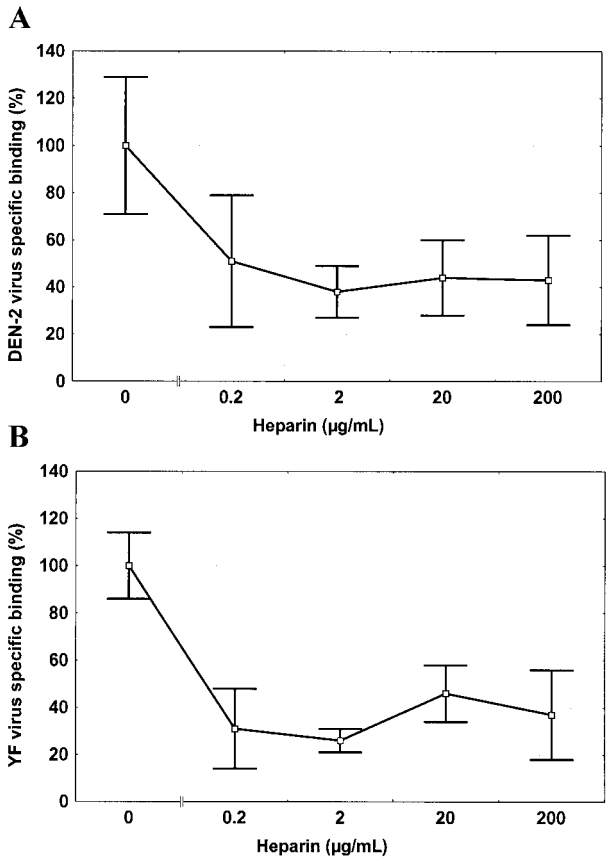

FIG. 3. Effect of heparin on DEN-2 and YF virus binding to Vero cells. DEN-2 virus (A) or YF virus (B) suspension was treated with differing heparin concentrations before the binding assay was performed. Results are presented as percentages of untreated control ± standard deviation. The mean values shown correspond to a quadruplicate assay. This experiment was repeated two times with similar results.

ID_{50} values determined in this study for cell binding of DEN and YF viruses and for the infection by YF virus. The binding of YF virus to wild-type CHO K1 cells was too low to be detected. Moreover, DEN-2 virus binding to these cells was 80% lower than on Vero cells (results not shown). Chen *et al.* (1996) have shown that the recombinant envelope protein of DEN-2 virus binds three times more efficiently to Vero cells than to CHO cells. This could be due to a difference in the nature or density of HS on the surface of the two cell types or to a difference in secondary receptor types on the cells.

Apart from indirect entry into cells of DEN and YF viruses by the antibody-Fc receptor (Schlesinger and Brandriss, 1983; Brandt *et al.,* 1982; Wang *et al.,* 1995), several virus receptors have been described for YF and DEN-2 viruses. The French neurotropic strain of YF recognizes different receptors in mouse and monkey brains (Ni *et al.,* 2000). DEN-2 virus was found to bind to 80- and 67-kDa proteins on the membrane of an *Aedes albopictus* derived cell line C6/36 (Munoz *et al.,* 1998) to a 65-kDa protein on mouse neuroblastoma cells (Ramos-Castaneda *et al.,* 1997) and to a 100-kDa protein in human bone marrow (Rothwell *et al.,* 1996). The differences in the binding affinity and the cell surface density of these proteins could account for the viral tropism observed (Putnak *et al.,* 1997).

The interaction between viruses and GAGs seems to be complex and to depend on numerous factors. A study of HS as receptor for classical swine fever virus suggested that the interaction between GAGs and virus is dependent on the virus strain, the target cell, and the number of passages of the virus in cell culture. The binding of the virus to GAGs could be specific but could also be due to nonspecific electrostatic interactions. Results obtained *in vitro*, *ex vivo*, and *in vivo* are also different (Hulst *et al.,* 2000). Thus it would be important to perform *ex vivo* and *in vivo* studies on the interaction between HS and flaviviruses. Recently, a new animal model for flavivirus infection was established using the murine Modoc virus (Leyssen *et al.,* 2001). This system may provide information on the flavivirus infection process in general.

Our results with infectious DEN-2 and YF viruses show that the interactions between these viruses and cell surface GAGs are important for virus–cell attachment. HS may be required to concentrate virus particles at the cell surface and to facilitate binding to a second receptor specifically recognized by the viral envelope protein. Alternatively, HS may initiate changes in the envelope of the adsorbed virus particles that trigger the process of fusion with the cellular membrane. Other *Flaviviridae* may use the same mechanism to bind to target cells. Two potential GAG-binding motifs were determined on the E protein of Murray Valley encephalitis virus (genus *Flavivirus*) (Chen *et al.,* 1997), but other surface molecules are also used as receptors (Lee and Lobigs, 2000). Bovine viral diarrhea virus (BVDV, *Pestivirus* genus) binds also to HS but additionally a 50-kDa protein has been

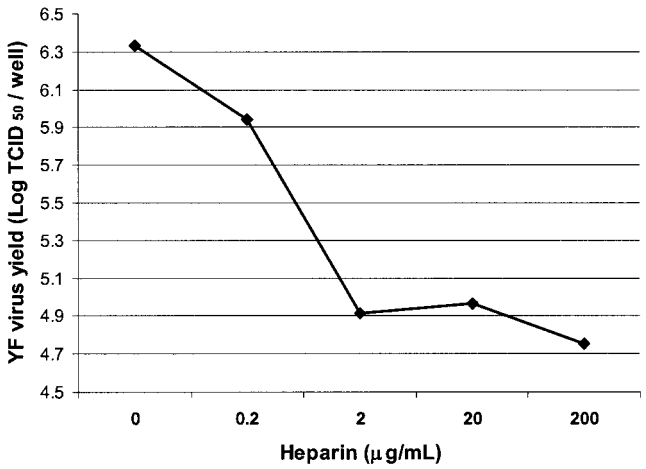

FIG. 4. Effect of heparin on YF virus replication. Cells were incubated for 48 h with YF virus in the presence of various heparin concentrations, washed, frozen, and thawed before virus titer determination. The mean values of the virus titers correspond to a quadruplicate assay.

identified as a putative BVDV receptor (Zheng *et al.,* 1998; Iqbal *et al.,* 2000). The HCV E2 envelope glycoprotein seems to interact with GAGs (Meyer *et al.,* 2000; Takikawa *et al.,* 2000) but this virus may have multiple receptors (Germi *et al.,* 2001). Therefore, infection by HCV may have similar features as infection by DEN and YF viruses.

MATERIALS AND METHODS

Cell culture

The following adherent cell lines were used: African green monkey kidney Vero cell line (ATCC CCL-81); wild-type Chinese hamster ovary cells (CHO K1); and mutant CHO cells, CHO psgB-618 ATCC CRL 2241 (defective in galactosyltransferase I) and CHO psgD-677 ATCC CRL 2244 (defective in HS polymerization). Vero cells were grown in M199 medium and CHO cells in F12K Ham's medium supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C with 5% CO_2. All media and FCS were from Life Technologies SARL, Cergy Pontoise, France.

For all the experiments described below (GAG-lyases, chlorate ± sulfate, heparin) the viability of the cells was tested by trypan blue staining. Cell viability was not affected significantly by any of the treatments (Student's *t* test: $P > 0.05$).

Viruses

Dengue virus type 2 strain New Guinea C and Yellow fever virus strain 17D viruses were propagated in Vero cells grown in M199 FCS-free medium. The culture supernatants were collected, centrifuged to eliminate remaining cells, aliquoted, and frozen at −80°C.

DEN-2 virus and YF 17D virus binding and infectivity assays

Cells were distributed to 24-well plates and grown to confluence (one million cells per well). The cells were then washed twice with FCS-free medium and incubated for 15 min at 4°C with DEN-2 virus ($10^{4.6}$ $TCID_{50}$/well) or YF virus ($10^{4.9}$ $TCID_{50}$/well). Unbound virus was removed by washing each well three times with culture medium. Then the cells, in 1 mL of medium, were frozen at −80°C and thawed at 37°C three times to release bound virus. Four hundred microliters (8 × 50 μL) of the clarified virus suspension was used for virus titration on Vero cells (96-well plates). The virus titer was determined using the method described by Reed and Muench (1938). For each experiment, four independent assays were performed. When the binding assay was carried out on CHO cells, the infectious titer was established using Vero cells because of the lack of cytopathic effect of DEN-2 virus on CHO cells. As a control, virus that remained associated with the cell debris after freezing/thawing was titrated after mixing the membrane pellet with 400 μL of medium. Less than 14 $TCID_{50}$/well, which represents less than 1% of the virus released by freezing and thawing after the binding assay, remained associated with the cell debris.

GAG-lyase treatment

Before the binding assay, confluent Vero cells were washed and incubated 1 h at 37°C with 300 μL of lyase buffer (10 mM phosphate buffer, pH 7.4, 0.14 M NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% glucose, 0.5% bovine serum albumin (Sigma, St. Quentin-Fallavier, France), 1% FCS) containing 0.2 IU/mL of chondroitin ABC lyase (Sigma) or 8 mIU/mL of heparin-lyase I (Sigma). Cells were washed with cold FCS-free medium before inoculation with virus.

Inhibition of sulfation

Before the binding assay, Vero cells were grown to confluence, washed with sulfate-free MEM Dulbecco's medium (Life Technologies SARL), and maintained 48 h in the same medium supplemented with 10% of dialyzed FCS in the presence of 30 mM of the sulfation inhibitor sodium chlorate ($NaClO_3$, Sigma). Cells were removed from their culture surface, distributed to 24-well plates, and grown to confluence (48 h in the same sulfate-free medium supplemented with sodium chlorate). For the reversal of sulfation inhibition, chlorate-treated cells were supplemented with 10 mM sodium sulfate (Na_2SO_4, Sigma).

Heparin and chondroitin sulfate interaction with DEN-2 and YF viruses

DEN-2 virus ($10^{4.6}$ $TCID_{50}$/well) or YF virus ($10^{4.9}$ $TCID_{50}$/well) was incubated for 1 h at 37°C with varying concentrations of heparin (0–0.2–2-20–200 μg/mL) or with 200 μg/mL of chondroitin sulfate B (Sigma). For the virus-binding study, the heparin- or chondroitin sulfate B-treated virus suspensions were used as described in the binding assay.

For the YF virus infectivity study, the heparin-treated virus suspensions ($10^{3.6}$ $TCID_{50}$/well) were incubated with the cells for 48 h. Then cells were washed three times to eliminate heparin. The cells with 1 mL of medium were frozen at −80°C and thawed at 37°C three times to release viruses. The virus yield was titrated by Reed and Muench method.

Statistical evaluation

All results are expressed as means ± standard deviations. The error bars in the graphs represent standard deviations. Results were tested for significance using Student's *t* test and the Spearman test (Statistica kernel version 5.5, Statsoft, Maisons-Alfort, France). Results that had *P* values < 0.05 were considered significant.

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